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MINIREVIEW

Identification and Mapping of Single Nucleotide Polymorphisms in the Varicella-Zoster Virus Genome

Benjamin Faga,* Wendy Maury,* David A. Bruckner,† and Charles Grose*,[1]

*Department of Microbiology, University of Iowa, Iowa City, Iowa 52242; and †Division of Laboratory Medicine, University of California at Los Angeles, Los Angeles, California 90049

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The primordial varicella-zoster virus (VZV) is estimated to have emerged 70 million years ago (McGeoch and Davison, 1999). Even with this ancient history, VZV has long been considered to have a stable genome. The complete DNA sequence (124,884 bp) was published by Davison and Scott in 1986. The VZV genomic sequence with its 69 open reading frames (ORFs) is frequently cited, even for strains other than Dumas, with the presumption of identity. The recent discovery of a genetically variant virus has led us to reconsider the presumption of VZV immutability (Santos *et al.,* 1998). In the case of VZV-MSP, we discovered a virus with a missense mutation in a major glycoprotein called gE. Because this point mutation eliminates a B cell epitope, we consider this polymorphism to have been the likely end result of an antibody selection in an infected human host (Grose, 1999). This mutant virus exhibited an accelerated cell-to-cell spread phenotype in both infected cells and the SCID-hu animal model of VZV infection (Santos *et al.,* 2000). Prior to this observation, all VZV wild-type isolates and strains were considered to have a phenotype so similar as to be indistinguishable.

Based upon its likely pathogenesis, VZV undergoes only about 20 cycles of replication during primary infection (Grose, 2000); even though this period provides limited opportunity for genetic diversification, some changes obviously occurred to generate VZV-MSP. Therefore, we postulated that a slowly coevolving VZV genome could be investigated by a similar genetic approach as directed toward the human genome (Collins *et al.,* 1998). The Human Genome Project has concentrated enormous effort over the past 3 years on the identification of single nucleotide polymorphisms (SNPs), the most common type of genetic variation. SNPs are sites that contain single base pair variations (Collins *et al.,* 1997, 1998). These sites generally are considered biallelic due to the limited number of transversions that have been found (Brookes, 1999). Each allele must occur in more than 1% of the population to be considered an SNP (Kruglyak *et al.,* 1997; Wang *et al.,* 1998). Individuals who share many of the same SNPs are likely to have arisen from a common ancestor and can therefore be grouped by inheritance (Collins *et al.,* 1997). Geneticists have estimated that a comparison of the corresponding nucleotide sequences from as few as 8–10 individuals can provide valuable information about the frequency and location of SNPs (Collins *et al.,* 1998; Wang *et al.,* 1998).

To assess whether VZV-MSP represented a truly unusual VZV variant genome, we selected 10 geographically diverse VZV strains and isolates for this analysis. VZV-MSP was isolated in Minnesota in late 1995 (Santos *et al.,* 1998). VZV-32 was isolated in Texas in the 1970s (Grose, 1980). VZV-Oka was isolated in Japan and attenuated in the 1970s (Takahashi *et al.,* 1975); the vaccine virus was a gift from M. Takahashi. VZV-VSD was a wild-type virus collected in South Dakota in the 1980s. VZV-VIA was isolated in Iowa from a child with chickenpox in the 1990s. VZV-Iceland was isolated in Iceland from vesicle fluid of a child with chickenpox in the 1990s. VZV-Ellen was originally isolated in Georgia from a child with chickenpox in the 1960s and obtained from the American Type Culture Collection (Gershon *et al.,* 1973). VZV 80-2 was originally isolated in Pennsylvania from an adult with herpes zoster in the 1980s (Ecker and Hyman, 1982). Two viral isolates, VZV-LAX1 and VZV-LAX2, were collected in Los Angeles in the early 1990s, before the introduction of varicella vaccine. The methods for VZV propagation in cell culture, as well as the methods for PCR amplification and DNA sequencing, have been described in detail (Santos *et al.,* 2000).

[1] To whom correspondence and reprint requests should be addressed at University of Iowa Hospital/2501 JCP, 200 Hawkins Drive, Iowa City, IA 52242. Fax: 319-356-4855. E-mail: charles-grose@uiowa.edu.

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VZV-MSP was sequenced at passage 8, VZV-32 at passage 18, VZV-VSD at passage 5, VZV-VIA at passage 9, VZV-Iceland at passage 9, VZV 80-2 at passage 10, VZV-LAX1 and VZV LAX2 at passage 6, VZV-EIlen at passage >100, and VZV-Oka vaccine strain at passage 30. All sequences were compared with that of VZV-Dumas (Accession No. XO4370); VZV-Dumas was isolated in The Netherlands in the 1970s but its passage history was not detailed (Davison and Scott, 1986).

Six genes were sequenced in each of the 10 strains. The noncoding and coding polymorphisms are listed in Table 1. The VZV gE gene was of particular interest because of the recent discovery of the gE escape mutant strain VZV-MSP (Santos *et al.,* 1998). Surprisingly, five gE polymorphisms were found among the 10 tested strains and isolates, 4 of which caused amino acid substitutions. VZV-EIlen, VZV-Iceland, and VZV 80-2 had three identical polymorphisms. One was a synonymous mutation within codon 220 (bp 660). Two nonsynonymous mutations in these three strains caused amino acid substitutions within codons 40 (T → I) and 536 (L → I). VZV-LAX1 and the vaccine strain VZV-Oka also contained the mutation within codon 40 but lacked the other two mutations found within VZV-EIlen, VZV-Iceland, and VZV 80-2. VZV-VSD was the only strain tested which contained a polymorphism within the gE cytoplasmic domain of gE. Interestingly, this change within codon 603 (G → D) inserted an additional acidic amino acid adjacent to the acidic casein kinase II phosphorylation site of gE (Grose, 1990; Olson *et al.,* 1997; Zhu *et al.,* 1996). VZV-LAX2, VZV-32, and VZV-VIA were the only strains tested that did not contain gE substitutions when compared to the Dumas strain. Since the codon 150 (D–N) mutation previously found in VZV-MSP gE was not discovered in any other strain, VZV-MSP gE retained a unique sequence among all currently tested strains and isolates.

The glycoprotein genes B and L showed a remarkable consistency of sequence in that 8 of 10 strains were identical to VZV-Dumas (Davison and Scott, 1986). Only VZV-Oka and VZV-LAX1 contained any polymorphisms and these polymorphisms were identical between the two strains. In gL, two changes were discovered; the first included the insertion of a methionine codon between amino acids 9 and 10 (bp 27 and 28), which is of great interest because it may represent an alternative start site. Second, there was a G to A substitution at bp 319 (when compared with VZV-Dumas) that resulted in the conversion of an alanine to a threonine residue. These differences together with the polymorphisms in the gI and gH genes are highlighted in Table 1.

The fact that polymorphisms were easily discernible in the major glycoprotein structural genes led us to examine whether a similar situation existed in the major VZV regulatory gene called IE62 (Perera *et al.,* 1992, 1993; Moriuchi *et al.,* 1994). Overall, 40 polymorphisms were found in the IE 62 gene of the 10 tested strains, when compared to the VZV-Dumas IE 62 gene (Table 1). As was the case with the gE and gH gene sequences, VZV-EIlen, VZV-Iceland, and VZV 80-2 showed remarkable similarity in their IE 62 genes. VZV-Oka and VZV-LAX1 were similar to these 3 strains, as they shared the silent IE 62 substitutions within codons 61 (bp 183) and 129 (bp 387) but lacked the change at codon 1071 (bp 3211). VZV-Oka shared additional polymorphisms with VZV-EIlen at codons 341 (bp 1023) and 958 (R → G) that were not present in VZV-Iceland and VZV 80-2. VZV-EIlen revealed eight unique substitutions in addition to those shared with VZV-Iceland, VZV 80-2, and VZV-Oka. VZV-Oka and VZV-LAX1 exclusively shared four polymorphisms at codons 473, 509, 609, and 1274 (bp 1419, 1527, 1827, and 3822, respectively).

Both VZV-32 and VZV-VIA contained identical polymorphisms within IE 62 at codons 516 (bp 1548), 1057 (Q → R), 1072 (Q → R), 1080 (bp 3240), and 1241(S → G). VZV-32 also contained five unique changes and the R958G mutation found in VZV-EIlen and VZV-Oka. VZV-VIA revealed four unique polymorphisms, which combined with the unique changes within VZV-32 allowed differentiation of VZV-32 and VZV-VIA. VZV-VSD contained one unique substitution (A → V at codon 602) but otherwise was identical to the Dumas strain. VZV-MSP as well as VZV-LAX2 contained no IE62 polymorphisms, other than the synonymous substitution found within codon 30 (bp 90), when compared to the Dumas strain.

For completeness, transitions were compared with transversions. Mutations that result in the substitution of a pyrimidine for a pyrimidine or a purine for a purine are called transitions. Substitutions of a purine for a pyrimidine and vice versa are transversions. Transversions are much less common than transitions in the human genome. When the numbers of transitions and transversions were counted for the sequenced VZV genes, 77% were transitions. Interestingly, all three substitutions in gB were transversions. This result was in contrast with the other genes. For example, the gE gene had 1 transversion and 4 transitions; the IE62 gene had 6 transversions and 34 transitions.

The DNA encoding the structural glycoprotein genes gE, gH, gB, gI, and gL as well as the immediate early gene 62 accounted for nearly 13,000 nucleotides. When the complete nucleotide sequences for the aggregate total of 60 genes were compared with their counterparts in the VZV Dumas strain, 61 polymorphisms were observed. Within the glycoprotein genes 21 polymorphisms were found, and within the IE62 gene 40 polymorphisms were found. A nucleotide difference found in only one virus may be unique to that virus and not found in any other virus, i.e., it may not represent a SNP. For this report, therefore, only alleles found in at least two viruses were considered SNPs. In other words, even if 98 additional as yet unsequenced VZV genomes lacked this particular change, it would still qualify as a SNP. Based

TABLE 1

VZV DNA

	Polymorphisms without amino acid change			Polymorphisms with amino acid change		
gene	number	mutation	strain	number	mutation	strain
gH (37)	3/10	t39g> nc	Ellen, Iceland, 80-2	9/10	c806t>P269L	MSP, Ellen, Iceland, Oka, 32, 80-2, VIA, LAX1, LAX2
		t2028c> nc	Ellen, Iceland, 80-2	5/10	g2099a>R700K	Ellen, Iceland, 80-2, Oka, LAX1
		c2181t> nc	Ellen, Iceland, 80-2	3/10	g215a>R72K	Ellen, Iceland, 80-2
	2/10	g2445a> nc	32, VIA			
		g573t> nc	Oka, LAX1			
	1/10	t1254g> nc	Ellen			
gE (68)	3/10	t660c> nc	Ellen, Iceland, 80-2	5/10	c119t>T40I	Ellen, Iceland, 80-2, Oka, LAX1
				3/10	c1606a>L536I	Ellen, Iceland, 80-2
				1/10	g448a>D150N	MSP
					g1808a>G603D	VSD
gI (67)	2/10	g546a> nc	Oka, LAX1	2/10	a15c>Q5H	32, VIA
gL (60)				2/10	27atg28 insert	Oka, LAX1
					g319a>A107T	Oka, LAX1
gB (31)	2/10	a294c> nc	Oka, LAX1	2/10	a217c>T73P	Oka, LAX1
		g390t> nc	Oka, LAX1			
IE62	10/10	g90c> nc	MSP, VSD, LAX2, Ellen, Iceland, 80-2, Oka, LAX1, 32, VIA	3/10	t2108c>V703A	Ellen, Iceland, 80-2
	5/10	c183t> nc	Ellen, Iceland, 80-2, Oka, LAX1		a2872g>R958G	Ellen, Oka, 32
		t387c> nc	Ellen, Iceland, 80-2, Oka, LAX1		g1969a>A657T	80-2, Oka, LAX1
	3/10	c3211a> nc	Ellen, Iceland, 80-2	2/10	a3170g>Q1057R	32, VIA
	2/10	a1023g> nc	Ellen, Oka		a3215g> Q1072R	32, VIA
		a1419g> nc	Oka, LAX1		a3721g>S1241G	32, VIA
		g1527t> nc	Oka, LAX1	1/10	a583g>N195D	Ellen
		a1827g> nc	Oka, LAX1		a3728g>E1243G	Ellen
		t3822c> nc	Oka, LAX1		t3763g>S1255A	Ellen
		g1548a> nc	32, VIA		t124g>S42A	Iceland
		a3240g> nc	32, VIA		a1882g>S628G	Oka
	1/10	a393g> nc	Ellen		t3622g>S1208A	Oka
		t516c> nc	Ellen		t3683c>L1228P	Oka
		a570g> nc	Ellen		c1805t>A602V	VSD
		t2887c> nc	Ellen		a3602g>H1201R	32
		a3279g> nc	Ellen		a3644g>Q1215R	VIA
		a3429g> nc	Oka		a3778g>I1260V	VIA
		a2064g> nc	VIA			
		a3435g> nc	VIA			
		t2229c> nc	32			
		a2637g> nc	32			
		a3105g> nc	32			
		a3624g> nc	32			

Note. The VZV genes are designated numerically according to the Davison and Scott (1986) nomenclature. The nucleotide numbers begin at the start site of each respective open reading frame; similarly, the amino acids are enumerated from the first methionine within each open reading frame. nc, no change; insert, new atg site.

SNP Groupings

	Dumas	MSP	VSD	LAX2	Ellen	Iceland	80-2	Oka	LAX1	32	VIA
Dumas	X	33	34	33	19	21	20	15	17	25	26
MSP	33	X	33	34	20	22	21	16	18	26	27
VSD	34	33	X	33	19	21	20	15	17	25	26
LAX2	33	34	33	X	20	22	21	16	18	26	27
Ellen	19	20	19	20	X	32	31	14	12	14	13
Iceland	21	22	21	22	32	X	33	12	14	14	15
80-2	20	21	20	21	31	33	X	13	15	13	14
Oka	15	16	15	16	14	12	13	X	32	10	9
LAX1	17	18	17	18	12	14	15	32	X	10	11
32	25	26	25	26	14	14	13	10	10	X	33
VIA	26	27	26	27	13	15	14	9	11	33	X

FIG. 1. Classification of VZV on the basis of SNP analysis. A polymorphism must be found in at least 2 of 10 strains to be called a SNP. Based on this definition, a total of 34 SNPs were delineated after genotyping of six different ORFs of 10 VZV strains. Subgroups of VZV strains (as defined by at least 85% shared SNPs) are shown within the four boxes. The strains from Japan, Iceland, and The Netherlands segregated into different clusters. GenBank accession numbers: VZV-MSP (AY005330-5), VZV-Ellen (AY010902-7), and VZV-32 (AF314215-21).

upon these criteria, among all polymorphisms detected, 34 were considered SNPs.

As proposed in the Human Genome Project, shared SNPs facilitate grouping by likely common ancestry. In the human genome, all SNPs are considered to have originated after divergence from the great apes but before the emergence of different human populations; by the concept of coevolution of herpesviruses with humankind, therefore, VZV SNPs presumably emerged during a similar period of geologic time (Brookes, 1999). When the shared SNPs among the 10 VZV strains were tabulated, any two viruses that shared at least 85% (29/34) SNP genotypes were considered to be closely related. As can be clearly seen, four groups have emerged by this analysis (Fig. 1). Of particular interest, the viruses from The Netherlands, Iceland, and Japan segregated into different groups. The VZV-Oka vaccine strain clustered with one isolate from Los Angeles and presumably represented polymorphisms of Japanese/Asian ancestry. VZV-Ellen, VZV-Iceland, and VZV 80-2 formed another group; these viruses were isolated from Iceland and two states along the Atlantic coast of the United States. VZV-32 and VZV-VIA formed a third group from the central United States. Of great relevance, VZV-MSP was most closely related to the VZV-Dumas prototype strain and was included in a fourth group along with VZV-VSD and LAX2; this VZV group presumably originated from Northern Europe. Since the D150N change in the gE gene occurred only in VZV-MSP, by our definition it was not a SNP. In fact, it was most likely a rare contemporary mutation in the VZV genome.

The SNP analysis facilitated the identification of "hot spots" within individual ORFs (Wang *et al.,* 1998). One example, in particular, was gH, where an amino acid substitution (P269L) was found within VZV-MSP gH. The same gH P269L polymorphism was also detected within most of the VZV strains tested. Because of this unexpected finding, we performed a BLAST peptide search coupled with MaxHomology alignment to assess which amino acid was present in the homologous regions of gH within other alphaherpesviruses (Fig. 2) (Rost, 1996; Sander and Schneider, 1991; Karlin and Aschtul, 1993). Interestingly, the substitution of leucine at codon 269 (or its homolog) leads to greater identity with gH found in different strains of pseudorabies virus as well as bovine herpes virus type 1. Other alphaherpesviruses, including herpes simplex virus, turkey herpes virus, and equine herpes virus type 1, contained a proline at the homologous site. Simian varicella virus was the only strain examined which did not express either a proline or leucine at this site but instead contained a serine residue. Therefore, a proline or leucine residue is present within this site on the gH ectodomain not only within different VZV strains but also within the homologous region of many alphaherpesviruses (Fig. 2).

Finally, the VZV SNP analysis mimics human DNA in that it was not predictable which viral ORF would exhibit mutations (Collins *et al.,* 1997; Wang *et al.,* 1998). Under certain circumstances, the absence of SNPs may suggest a highly conserved essential function of the ORF. For example, the low number of SNPs in the relatively small gI or gL genes supported an earlier concept that both gene products behave as chaperone proteins, gI with gE and gL with gH (Duus and Grose, 1996; Mallory *et al.,* 1997). On the other hand, the large number of polymorphisms within the 10 SNP profiles of IE62 was remarkable. These data reaffirm the recent observations by others that VZV-Oka vaccine strain IE62 exhibited several polymorphisms (Argaw *et al.,* 2000; Gomi *et al.,* 2000); our IE62 sequence differed in turn from those in the other two publications. The data also emphasize that wild-type strains exhibit IE62 polymorphisms not found in VZV-Dumas. In this situation, SNP analysis is a powerful tool by which to assess whether IE62 has a role in VZV attenuation; presumably any IE62 polymorphism likely to be involved in attenuation would be unique to VZV-Oka

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VZV-MSP      G LP IELIVVP HTVKLNAVTS DTTWFQLNPP GPDPGPSYRV YLLGRGLDMN
vzvd         G PP IELIVVP HTVKLNAVTS DTTWFQLNPP GPDPGPSYRV YLLGRGLDMN
ch25806      G SP IEILVVP HTVNLAAITS NT-WFQYNPP GPDPGPTYQV HILGRGVHDN
hsve4        N PP LEMIVAP NDVRARIVNR LPPRRRLEPP GPYAGPIYKV YVLSDGLGHG
hsveb        N PP LEMIVVP NDVSARILNR RPSRLRLEPP GPHAGPIYKV YVLSDGLGHG
hsvbc        L LP LEFMVAP ADANVRMITA FNGGGAFPPP GPAAGPQRRA YVIGYGNSRL
prvri        F LP LEVIISA ERMRM..IAP PALGAGLEPP GPPAGR.FHV YTLGFLSDGA
prvn3        F LP LEVIISA ERMRM..IAP PALGADLEPP GPPAGR.FHV YTLGFLSDGA
prvka        F LP LEVIISA ERMRM..IAP PALGSDLEPP GPPAGR.FHV YTLGFLSDGA
hsv1e        S PP AEVMVVP AGQTLDRVGD PDENPPGALP GPPGGPRYRV FVLGSLTDNG
hsv11        S PP VEVMVVP AGQTLDRVGD PDENPPGALP GPPGGPRYRV FVLGSLTDNG
hsvtu        N PP IELIISA KYRNLSLLWP ..PRQQYEPV NKGTGRPHWI YLLGVYRNVS
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FIG. 2. Comparison of VZV gH at codon 269 to homologous gH regions of other alphaherpesviruses. Blast peptide and MaxHom searches were performed at the Predict Protein Website (Columbia University, http://dodo.cpmc.columbia.edu/predictprotein/). The VZV gH proline–proline sequence at codons 269–270 was compared to the homologous region on the listed alphaherpesviruses. Note that all strains except simian varicella virus contained either a proline or leucine at the homologous Pro269 codon of VZV gH. VZV-MSP and vzvd, varicella-zoster virus; ch25806, simian varicella virus; hsve4 and hsveb, equine herpes virus; hsvbc, bovine herpes virus; prvri, prvn3, and prvka, pseudorabies virus; hsvle and hsv11, herpes simplex virus; hsvtu, turkey herpes virus.

vaccine IE62 and not present in any wild-type strain, i.e., the polymorphism would not be a SNP (Table 1). Interestingly, SNP analysis in the human genome suggests that polymorphisms with C ↔ T substitutions may reflect potential cytosine methylation sites, since cytosine residues within CpG dinucleotides are the most mutable sites within the human genome (Wang *et al.,* 1998). CpG methylation certainly remains an unexplored mechanism in a VZV regulatory gene.

Furthermore, the SNP data may be relevant to VZV pathogenesis. Within the human genome, one SNP in one gene can serve as a marker for a specific genetic disease. By extension, the VZV SNP data indicated a genetic potential for an occasional emergence of a wild-type virus with polymorphisms which either increase virulence or delete B or T cell epitopes present in the vaccine virus. In this regard, it is noteworthy to point out that the VZV-MSP virus is called an escape mutant because it lacks an epitope found in the gE virion surface protein of most wild-type strains as well as the vaccine strain VZV-Oka. Likewise, the mutable IE62 protein is known to harbor T cell epitopes which remain largely unmapped; these epitopes could include a SNP (Arvin *et al.,* 1986; Bergen *et al.,* 1991). As part of this SNP analysis, the discovery that two randomly selected VZV isolates from Los Angeles differed considerably in their SNP profiles (Table 1 and Fig. 1) suggested a very heterogeneous VZV population in the Los Angeles area. Therefore, we recommend SNP analysis during unexpected future outbreaks of chickenpox (Buchholz *et al.,* 1999), in order to better define the viral genotype.

In summary, SNP analysis of the VZV genome provides an alternative approach to more traditional phylogenetic analysis. In fact, when a VZV evolutionary tree was constructed using the IE62 gene sequences, the phylogram (Fig. 3) was remarkably similar to the SNP groupings in Fig. 1. The SNP data and GrowTree program not only verify that VZV has continued to evolve in the 20th century but also document a previously unappreciated but intriguing genetic diversity of this reclusive human herpesvirus. For example, detection of one or two SNPs of the entire 124,884-bp genome now appears sufficient to

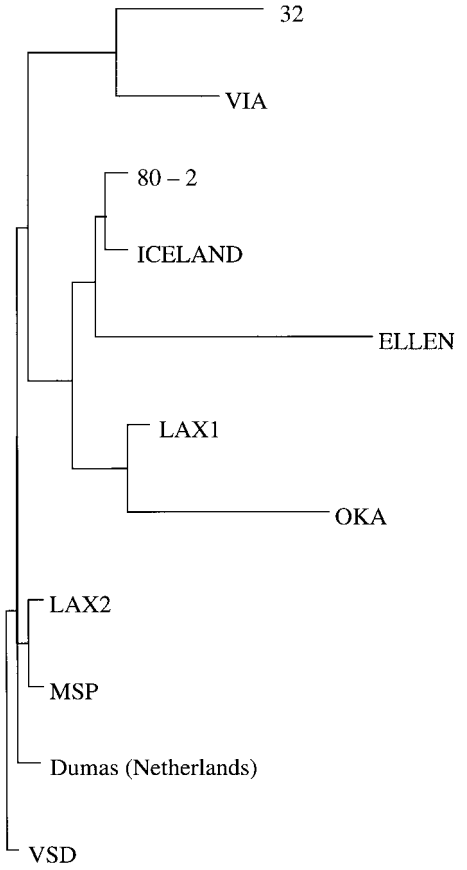

FIG. 3. Evolutionary analysis of the VZV IE62 gene. An evolutionary tree was generated using neighbor-joining analysis within the GrowTree program supplied by Genetics Computer Group, University of Wisconsin (Madison, WI). The Jukes–Cantor method was selected for distance correction.

classify VZV into subgroups. In this regard, identification of biologically important VZV SNPs and hot spots will facilitate the incorporation of relevant oligonucleotides into biochips for rapid microarray analysis of VZV mutations within large viral populations (Brown *et al.,* 1999; Zhu *et al.,* 1998). Based upon our experience with VZV-MSP and these SNP analyses, we predict the discovery of additional VZV genotypes with distinguishable phenotypes.

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